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Reaction cell inductively coupled plasma mass spectrometry-based immunoassay using ferrocene tethered hydroxysuccinimide ester as label for the determination of 2,4-dichlorophenoxyacetic acid

An-Ping Deng [a], Hui-Tao Liu [b], Shiuh-Jen Jiang [a,*], Hsuan-Jung Huang [a,1], Chi-Wi Ong [a]

[a] *Department of Chemistry, National Sun Yat-Sen University, Kaohsiung 80424, Taiwan*
[b] *Department of Chemistry, Lanzhou University, Lanzhou 730000, China*

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Abstract

The high sensitive dynamic reaction cell inductively coupled plasma mass spectrometry (DRC ICP-MS)-based immunoassay using ferrocene (Fc) tethered hydroxysuccinimide ester as label for the determination of 2,4-dichlorophenoxyacetic acid (2,4-D) was presented. Ferrocene tethered hydroxysuccinimide ester was directly or via horse radish peroxidase (HRP) as a bridge coupled to monoclonal antibody (mAb) of 2,4-D. Competitive immunoreactions were completed in microtiter plate and the signal of ^{56}Fe in the bound ferrocene labeled conjugate was detected by DRC ICP-MS. The potentially interfering $^{40}Ar^{16}O^+$ at the iron mass *m/z* 56 was reduced in intensity significantly by using NH_3 as the reaction gas. The optimization process of the immunoassay was greatly simplified in the aid of enzyme linked immunosorbent assay (ELISA) procedure. The 2,4-D was determined in the dynamic range of 0.1–1000 ng/ml and the detection limits of the assays using the two ferrocene labeled conjugates were found to be 0.044 and 0.055 ng/ml, respectively. The relative standard deviation for six measurements were within 5.5–15.3%.

Keywords: Immunoassay; Inductively coupled plasma mass spectrometry; Ferrocene labeled conjugate; 2,4-Dichlorophenoxyacetic acid

1. Introduction

Within the existing isotopic immunoassays such as enzyme linked immunosorbent assay (ELISA), chemiluminescent immunoassay (CLIA), fluorescent immunoassay (FIA), etc. for samples of clinical, pharmaceutical, biological and environmental importance, ELISA is no doubt the most frequently applied immunoassay due to its rapidity, simplicity and capability for multiple sample analysis. In recent years, more and more interests are attracted to incorporate ELISA microtiter plate with other sensitive detection techniques such as amperometry in flowing injection system [1–4] or inductively coupled plasma mass spectrometry (ICP-MS) for immuno-analysis [5–7].

* Corresponding author. Fax: +886-7-5253908.
E-mail addresses: sjjiang@mail.nsysu.edu.tw (S.-J. Jiang), hjhuang@mail.nsysu.edu.tw (H.-J. Huang).
[1] Co-corresponding author.

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ICP-MS is a powerful method for trace element determination because of its extremely high sensitivity and element specificity. However, the excellent performance of ICP-MS mainly focus on the field of inorganic element analysis [5]. In principle, if a compound containing a proper metal element is able to couple with the biological molecules such as antibodies, a corresponding ICP-MS-based immunoassay may be constructed. In biological and clinical analysis, colloidal Au particles have been extensively used to visualize protein structure in the cell and to detect receptor–ligand binding by electron microscopy [8]. In time-resolved fluoro-immunoassay (TR-FIA), lanthanide ions are complexed to a wide spectrum of molecules, and the fluorescence of their chelates provides a sensitive means of detection [9,10]. The ICP-MS-based immunoassays using colloidal Au particles and lanthanide chelates as biological labels have been reported [5–7].

Horse radish peroxidase (HRP) catalyzing the reaction between H_2O_2 and cosubstrate molecules is the enzyme label widely used in immunoassays. Depending on different cosubstrate molecules used, the enzyme activity can be determined by measuring the absorbance in the visible region, by chemiluminescence or by electrochemistry. As HRP contains Fe element in its active center, the amount of HRP bound on the solid support after immunoreaction may be detected directly by ICP-MS technique. However, unlike Au particles and lanthanide chelates as biological labels that provide hundreds of particles per antibody, the number of HRP molecules labeled on one antibody is usually in the range of 1–10. Consequently, the sensitivity of HRP-ICP-MS immunoassay will be lower. It is well known that ferrocene (Fc) or its derivatives containing Fe element in the center of dicyclopentadienyl have been widely used in electrochemistry as a mediator to enhance the electron transfer between electrode and solution [11–15]. It is possible to use ferrocene derivatives as biological labels to establish an alternative ICP-MS-based immunoassay. However, when a quadrupole analyzer is used, isobaric interferences due to various polyatomic ions can pose a problem and lead to erroneous results. It is well known that the accurate determination of Fe, which is enclosed in the active center in the most common enzyme HRP suffers seriously from the polyatomic spectroscopic interferences such as $^{38}Ar^{16}O^+$ and $^{40}Ar^{14}N^+$ on $^{54}Fe^+$, $^{40}AR^{16}O^+$ on $^{56}Fe^+$ and $^{40}Ar^{18}O^+$ on $^{58}Fe^+$. The dynamic reaction cell (DRC) and/or collision cell technique has proved to be an effective method for the alleviation of spectroscopic interferences in the ICP-MS analysis [16–22]. Tanner and Baranov [16] demonstrated that NH_3 could be used as the reagent gas to alleviate argon-based spectral interference in DRC ICP-MS analysis of Fe.

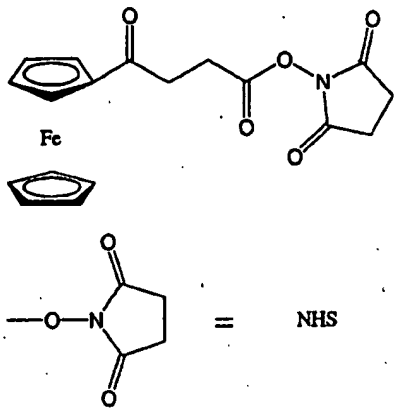

Fig. 1. Structure of ferrocene-$COCH_2CH_2CO$-NHS.

In this study, the attempt to establish a DRC ICP-MS-based immunoassay with NH_3 as reaction gas and ferrocene tethered hydroxysuccinimide ester (ferrocene-$COCH_2CH_2CO$-NHS, Fig. 1) as label was made for the determination of 2,4-dichlorophenoxyacetic acid (2,4-D), a common herbicide widely used in agriculture. Two Fc labeled conjugates were synthesized: (1) ferrocene-$COCH_2CH_2CO$-NHS was coupled directly to monoclonal antibody (mAb) of 2,4-D to form the conjugate of ferrocene-$COCH_2CH_2CO$-mAb (Fc-mAb); (2) ferrocene-$COCH_2CH_2CO$-NHS was firstly coupled to HRP to form ferrocene-$COCH_2CH_2CO$-HRP (Fc-HRP), then the Fc-HRP was coupled to mAb to produce the final conjugate of Fc-HRP-mAb. The indirect competitive immunoassay format for 2,4-D was adapted. The solid antigen coated on the wells of an ELISA microtiter plate was competed with 2,4-D in the solution for the Fc labeled conjugate. The bound conjugate was dissolved with 1% (v/v) nitric acid and determined by DRC ICP-MS. The more the 2,4-D added to the solution, the less is the Fe signal detected by DRC ICP-MS.

2. Experimental

2.1. Instrumentation

An ELAN 6100DRC ICP-MS instrument (Perkin-Elmer SCIEX, Thornhill, Ont., Canada) was used. Samples were introduced with an ESI 100 concentric nebulizer (ESI, Omaha, NE, USA). Polystyrene 96-well microtiter plates (MaxSorp and Polysorp) were from Nunc (Roskilde, Denmark). Multiple channel pipette was from Gilson (France). Milli-Q equipment for water purification was from Millipore (USA). The DRC ICP-MS conditions were selected that gave the minimum estimated detection limit of ^{56}Fe. Solutions of 1 ng/ml of Fe in pure water and 1% (v/v) HNO_3 (to be treated as blank) were introduced into the nebulization system successively. The aerosol generated was then transported to DRC ICP-MS for Fe detection. The operating conditions of the DRC ICP-MS used in this work are summarized in Table 1.

Table 1
Equipment and operating conditions

ICP-MS instrument	Perkin-Elmer SCIEX ELAN 6100DRC
ICP parameters	
rf power (W)	1200
Plasma gas flow (l/min)	15
Auxiliary gas flow (l/min)	1.13
Nebulizer gas flow (l/min)	1.02
Nebulizer	ESI 100 concentric, micro-flow
Sample uptake (μl/min)	100
DRC parameters	
NH_3 flow rate (ml/min)	0.4
Quadrupole rod offset (V)	−8
Cell path voltage (V)	−18
Cell rod offset (V)	−3
Rejection parameter *a*	0
Rejection parameter *q*	0.5
Autolens	On
Mass spectrometer settings	
Dwell time (ms)	50
Sweeps	20
Readings	1
Replicates	10
Isotope monitored	^{56}Fe

2.2. Reagents and solutions

Analytical-reagent grade chemicals were used without further purification. Ultrapure HNO_3 was obtained from Merck (Darmstadt, Germany). Iron and indium element standard solutions were from Fisher (Fair Lawn, NJ, USA). Reaction gas NH_3 (99.999% purity) was from Air Liquide (Taiwan). The mAb against 2,4-D (ascitic fluid, lyophilized, 0.45 ml per vial in distilled water, lot E2/G2) was a generous gift from Dr. Franek (Veterinary Research Institute, Brno, Czech Republic) [23]. Ferrocene-$COCH_2CH_2CO$-NHS was synthesized from 3-ferrocenyl-3-oxobutyric acid [24] via the acid chlorine and reacting with *N*-hydroxysuccinimide (NHS). HRP (Type VI-A, RZ 3.0), 3,3′,5,5′-tetramethylbenzidine (TMB), bovine serum albumin (BSA), casein, 2,4-D standard, *N*-hydroxysuccinimide, dicyclohexylcarbodiimide (DCC), dimethyl formamide (DMF) and Sephadex G-25 were purchased from Sigma (USA).

Coating buffer: 0.05 mol/l Na_2CO_3–$NaHCO_3$ solution, pH 9.6; assay buffer: 0.01 mol/l phosphate buffer saline (PBS), containing 0.5% casein and 0.15 mol/l NaCl, pH 7.4; the stock solution of 2,4-D (500 μg/ml) was prepared by dissolving 2,4-D powder with a small amount of DMF and then diluted with 0.1 mol/l PBS, pH 7.4. The standard solutions of 2,4-D (0.1, 1.0, 10, 100 and 1000 ng/ml) were prepared by diluting stock solution with assay buffer.

2.3. Synthesis of 2,4-D-BSA

The synthesis method was modified according to [25]. Briefly, 5.4 mg of 2,4-D, together with 2.8 mg of NHS and 5.1 mg of DCC were dissolved in 0.3 ml of DMF and kept on stirring overnight at room temperature. After centrifugation, half supernatant with the produced activated 2,4-D was slowly added to 0.8 ml of 0.13 mol/l $NaHCO_3$, pH 8.5 containing 50 mg of BSA. The reaction was performed at room temperature for 3 h with slight stirring. The mixture was desalted with a Sephadex G-25 column. Fractions containing the protein were pooled and dialyzed overnight at 4 °C against 0.013 mol/l $NaHCO_3$, pH 8.5. The synthesized 2,4-D-BSA conjugate was kept at −20 °C.

2.4. Synthesis of Fc labeled conjugates

(1) *Fc-mAb*: 0.27 mg of ferrocene-$COCH_2CH_2CO$-NHS (7.0×10^{-4} mmol) was dissolved in

20 μl DMF and added to 200 μl of 0.013 mol/l $NaHCO_3$ containing 2.3 mg of mAb against 2,4-D (1.4×10^{-5} mmol). The mixture was slightly stirred at room temperature for 3 h. After centrifugation, the suspension was applied to a Sephadex G-25 column. Fractions containing Fc-mAb were pooled and was kept at −20 °C until use.

(2) *Fc-HRP-mAb*: 0.62 mg of ferrocene-$COCH_2CH_2$CO-NHS (1.7×10^{-3} mmol) was dissolved in 27 μl DMF and added to 250 μl of 0.013 mol/l $NaHCO_3$ containing 2.5 mg of HRP (5.6×10^{-5} mmol). The mixture was slightly stirred at room temperature for 3 h and then dialyzed intensively against 0.013 mol/l $NaHCO_3$. The Fc-HRP solution produced in above step was oxidized with 85 μl of 0.1 mol/l $NaIO_4$ at room temperature for 20 min, and then dialyzed overnight in 1 mmol/l acetate buffer, pH 4.4. After centrifugation and pH adjustment with 0.1 ml of 0.05 mol/l carbonate buffer, pH 9.5, the supernatant was mixed with 100 μl of mAb (1.4×10^{-5} mmol) and kept on stirring for 3 h at 4 °C. Fifty microliters of 0.1 mol/l $NaBH_4$ was added to the mixture and the reaction was continued for another 2 h. Finally, the solution was going through a Sephadex G-25 column. Fractions containing Fc-HRP-mAb were pooled and was kept at −20 °C until use.

2.5. Immunoassay protocol

Competitive immunoassay format was employed for 2,4-D detection. Initially, 200 μl of 2,4-D-BSA (diluted with coating buffer at 1:5000) was pipetted into the wells of a microtiter plate and incubated at room temperature overnight. The unbound antigen was washed away with the assay buffer one time and incubated with 200 μl per well of assay buffer (containing 0.5% of casein) at room temperature for 1 h to block unspecific active sites. The blocking step not only reduced the unspecific binding of the Fe labeled conjugate on the wells, but also eliminated the unspecific adsorption of the Fe probably existed in the environmental samples on the wells. After washing the plate twice, 2,4-D standard solutions (100 μl per well) and Fc labeled conjugate (Fc-mAb or Fc-HRP-mAb, 100 μl per well) prepared with assay buffer were pipetted into the wells and incubated at room temperature for 1 h. The plate was washed three times with assay buffer, followed by another three times washing with pure water.

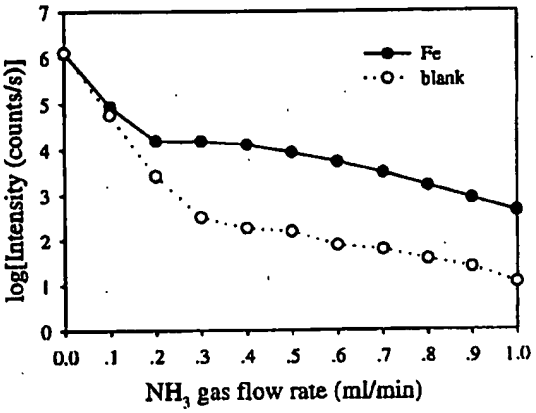

Fig. 2. Effect of NH_3 flow rate on the signal of 1 ng/ml Fe and blank at *m/z* 56. Rp*q* = 0.5, Rp*a* = 0.0.

External calibration was used for the quantitative determination of 2,4-D. Indium was used as internal standard to correct the fluctuation of the instrument. Two hundred microliters of 1% (v/v) HNO_3 solution containing 0.3 ng/ml In was added to each well. Samples to be analyzed were placed at room temperature for 15 min and then introduced to the DRC ICP-MS by a peristaltic pump.

3. Results and discussion

3.1. Operating conditions of DRC ICP-MS

NH_3 was selected as the reaction gas and two parameters affected the operation of the dynamic reaction cell the most, i.e. cell gas flow rate and the value of rejection parameter *q* (Rp*q*) of the DRC system were studied to get the best S/N value for ^{56}Fe. Fig. 2 shows the effect of the NH_3 gas flow rate on Fe and blank signals. As shown in Fig. 2, the blank signal at *m/z* 56 decreased rapidly with the increase of NH_3 gas flow rate. In contrast, NH_3 gas flow rate did not affect the signal of 1 ng/ml Fe significantly. After primary experiments a NH_3 flow rate of 0.4 ml/min and a *q* value of 0.5 were selected for the following experiment. Under these conditions, the estimated detection limit was 3.2 ng/l for Fe. Meanwhile, the rejection parameter *a*

(Rp*a*) did not affect ion signals when the value was less than 0.1. The Rp*a* value was set at 0 in this study.

3.2. Synthesis of Fc labeled conjugates

The synthesis of Fc-mAb is comparatively simpler as pre-activated ferrocene-$COCH_2CH_2CO$-NHS is able to couple with mAb in one reaction step. However, attention should be paid to avoid forming precipitate during coupling process. Using small amount of DMF to pre-dissolve ferrocene-$COCH_2CH_2CO$-NHS and keeping the starting mole ratio (ferrocene-$COCH_2CH_2CO$-NHS/mAb) to be 30–50 are suitable for the synthesis. As maximal UV spectrum peaks of ferrocene-$COCH_2CH_2CO$-NHS, mAb and Fc-mAb are overlapped at 280 nm, it is hard to calculate the final mole ratio in the synthesized Fc-mAb.

More reaction steps are required for the synthesis of Fc-HRP-mAb. Ferrocene-$COCH_2CH_2CO$-NHS was firstly coupled to HRP to form Fc-HRP, then the HRP in Fc-HRP conjugate was oxidized with $NaIO_4$ to form aldehydes with which mAb can be covalently coupled. The Schiff bases formed in the coupling process were thus reduced with $NaBH_4$ solution. The UV absorption spectra of ferrocene-$COCH_2CH_2CO$-NHS, HRP, Fc-HRP and Fc-HRP-mAb are shown in Fig. 3. According to the absorbance values of the compounds

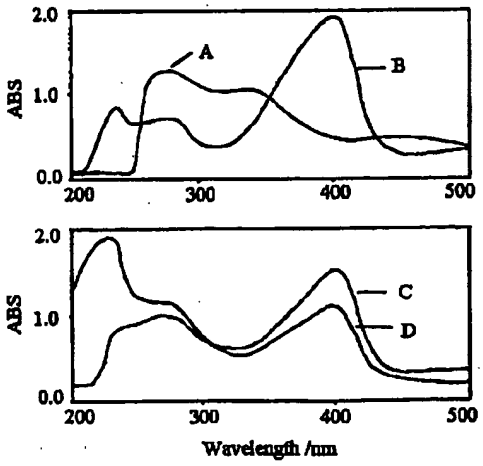

Fig. 3. Absorption spectra of ferrocene-$COCH_2CH_2CO$-NHS (curve A, 10^{-3} mol/l), HRP (curve B, 2×10^{-5} mol/l), Fc-HRP (curve C, 1:5 dilution) and Fc-HRP-mAb of 2,4-D (curve D, 1:5 dilution) in 0.013 mol/l $NaHCO_3$ with 1% of DMF.

appeared at 280 and 403 nm, the mole ratios of Fc/HRP and HRP/mAb were estimated to be 19 and 3.8, respectively.

3.3. Optimization of immunoreaction conditions

In the synthesized Fc-HRP-mAb, it was found that the enzyme activity remained was almost equal to that of HRP in free form. Thus, the optimization was simplified in the aid of ELISA procedure. The checkerboard titration method [26] was used in which serial dilutions of both coating antigen (BSA-2,4-D) and enzyme label (Fc-HRP-mAb) were incubated together. Two hundred microliters per well of BSA-2,4-D at the dilution of 1:1000, 1:2000, 1:5000 and 1:10,000 was added to the plate and kept at room temperature overnight. After blocking and washing steps, 100 μl per well of 2,4-D standards (concentrations of 0 and 100 ng/ml), together with 100 μl per well of Fc-HRP-mAb at the dilution of 1:1000, 1:2000, 1:5000 and 1:10,000 were added to the plate and incubated at room temperature for 1 h. Washing the plate and adding HRP substrate (TMB/H_2O_2) to the plate for enzymatic reaction 15 min, the colored solutions in the plate was measured by an ELISA reader. It was found that the maximum absorbance ratio at 0 and 100 ng/ml of 2,4-D were obtained when BSA-2,4-D and Fc-HRP-mAb were both at 1:5000. Thus, 1:5000 of BSA-2,4-D and 1:500 of Fc-HRP-mAb were selected in the corresponding ICP-MS-based immunoassay for the determination of 2,4-D in the dynamic range of 0.1–1000 ng/ml.

For the ICP-MS-based immunoassay with Fc-mAb as the conjugate, 1:5000 dilution of coating antigen was used and the series dilutions (1:200, 1:500, 1:1000) of Fc-mAb were tested. The maximum intensity ratio of Fe/In at 0 and 100 ng/ml of 2,4-D was obtained when the Fc-mAb was at 1:500. Therefore, 1:5000 of coating antigen and 1:500 of Fc-mAb were used in the assay.

3.4. 2,4-D determination

Under the optimal conditions, the 2,4-D standard solutions (0.1–1000 ng/ml) were applied to the ICP-MS-based immunoassays. After immunoreactions, the Fc labeled conjugate bound to the wells was dissolved by 1% (v/v) HNO_3 containing 0.3 ng/ml

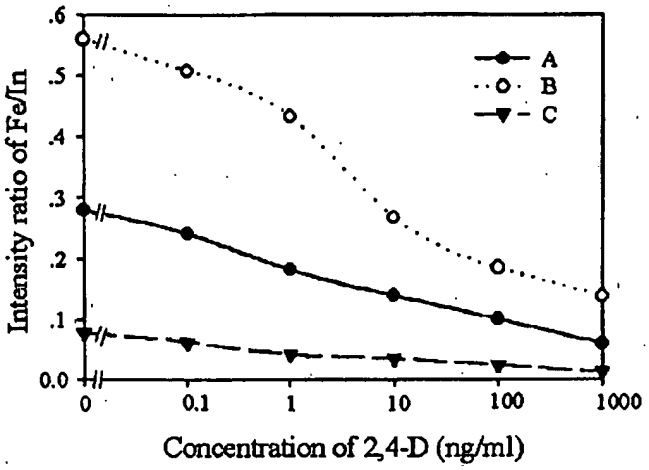

Fig. 4. Standard curves of DRC ICP-MS-based immunoassays for 2,4-D determination using Fc-HRP-mAb (curve A), Fc-mAb (curve B) and HRP-mAb (curve C) as conjugates.

of In and introduced to the ICP-MS system by a peristaltic pump. One percent (v/v) HNO_3 solution containing 0.3 ng/ml of In was used as blank solution and the intensity ratio of Fe/In was used as quantitative signal for the determination of 2,4-D. The standard curves for 2,4-D determination are plotted in Fig. 4. As shown in Fig. 4 (curves A and B), the detection limits (defined as producing 10% of inhibition) from ICP-MS-based immunoassays using Fc-HRP-mAb and Fc-mAb as conjugates were found to be 0.044 and 0.055 ng/ml, respectively, which were comparable to or even lower than those reported in electrochemical immunoassays for 2,4-D [25,27,28] and in ELISAs using HRP as enzyme label for 2,4-D [23]. The relative standard deviation for six measurements were within 5.5–15.3%. For comparison, the standard curve from the ICP-MS-based immunoassay using HRP-mAb as conjugate is also presented in Fig. 4 (curve C). It can be seen that the slope of curve C is much lower than those of curves A and B, indicating the lower sensitivity of the assay when only HRP was used as label.

4. Conclusions

The novel ICP-MS-based immunoassay using ferrocene-$COCH_2CH_2CO$-NHS as label for the determination of 2,4-dichlorophenoxyacetic acid was described. Ferrocene-$COCH_2CH_2CO$-NHS can be directly or using HRP as a bridge coupled to antibody to form the Fc labeled conjugates for the establishment of the assays. Although more coupling steps are required for the preparation of Fc-HRP-mAb, the optimization process of the assay was simplified. It was demonstrated that the sensitivity of these two ICP-MS-based immunoassays was within the same order of magnitude. The sensitivities of the presented assays for 2,4-D detection are equivalent to or even better than those in some immunoassays. Compared with other non-isotopic methods, the proposed detection has a wide choice of label and may expand the range of application, which would be a good starting for further experiments for real samples analysis.

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